Characterization of Alanyl-tRNA Synthetase Quality Control in *Bacillus subtilis*

Undergraduate Research Thesis

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By

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Abstract

Translation is an essential cellular process, resulting in the synthesis of proteins (1). While translation is critical for the cell, it is comparatively error-prone relative to other essential cellular processes. In translation, errors occur approximately once per 10^4 codons. In comparison, the error rate in DNA replication is approximately one error per 10^8 nucleotides (1). It is important to understand how these errors in translation affect the cell. Aminoacyl-tRNA synthetases (aaRSs) are critical enzymes in translation which ligate tRNA to their cognate amino acids. Due to their ability to edit tRNA mischarged with non-cognate amino acids through pre- and post-transfer editing, aaRSs have a role in prevention of errors in translation (1). A lack of aaRS quality control has been shown to be detrimental to the cell (2, 3). While aaRS quality control can be important for the cell, it has been observed that certain aaRSs can mischarge tRNA with non-cognate amino acids. Alanyl-tRNA synthetase (AlaRS) from *Streptococcus pneumoniae*, a Gram-positive organism, mischarges $tRNA^{Ala}$ with serine. Ser-$tRNA^{Ala}$ has been shown to be used by MurM, an enzyme involved in peptidoglycan crosslinking in *S. pneumoniae* (6). To further study AlaRS quality control in Gram-positive organisms, an AlaRS editing deficient *Bacillus subtilis* strain was made. *B. subtilis alaS*, which encodes for AlaRS, was cloned into pET28a, and mutagenized to encode C668A AlaRS (8). *B. subtilis* wild-type (WT) and C668A AlaRS were expressed and purified for characterization *in vitro* to confirm that the C668A mutation diminished AlaRS editing activity. WT and C668A AlaRS were able to charge $tRNA^{Ala}$ with alanine. However, C668A AlaRS was able to mischarge $tRNA^{Ala}$ with serine, and had a diminished ability to edit Ser-$tRNA^{Ala}$ compared to WT AlaRS. This suggests that C668 is an essential residue for AlaRS editing activity. Lastly, there was no significant difference in growth between the WT and AlaRS editing deficient *B. subtilis* strains in Luria Broth (LB) or M9 minimal media, which suggests that AlaRS quality control is not required for normal growth in these conditions.

Introduction

Translation is an essential cellular process, resulting in the synthesis of proteins. While translation is a critical cellular process, errors occur more frequently in translation than in other processes in the cell. The error rate of translation is approximately one error per 10^4 codons, while the error rate of DNA replication is approximately one error per 10^8 nucleotides (1). Since translation is a comparatively error prone cellular process, it is important to understand the effects of these errors on the cell.

Aminoacyl-tRNA synthetases (aaRSs) have an important role in translation; aaRSs attach amino acids to their cognate tRNAs (Figure 1). These enzymes are critical in the prevention of errors during protein synthesis, due to their ability to discriminate between cognate and non-cognate amino acids (1, 2). Since certain amino acids are structurally similar, aaRSs can perform pre- and post-transfer editing, to remove non-cognate amino acids from the aaRS or tRNA (Figure 2). The editing capabilities of aaRSs prevent errors in translation and highlight their importance in translational quality control (1, 2).

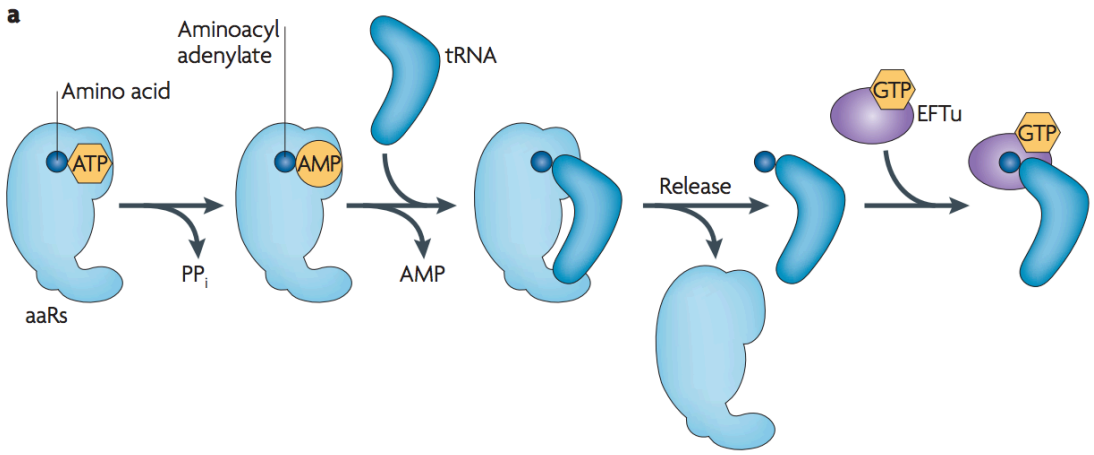

Figure 1: Aminoacyl-tRNA synthetases attach amino acids to the cognate tRNA, in a two-step reaction, resulting in ester bond formation. First, the amino acid and ATP are attached to the aaRS; ATP is required to generate the aminoacyl adenylate. Then, the amino acid is attached to tRNA, which results in an aminoacyl-tRNA (1).

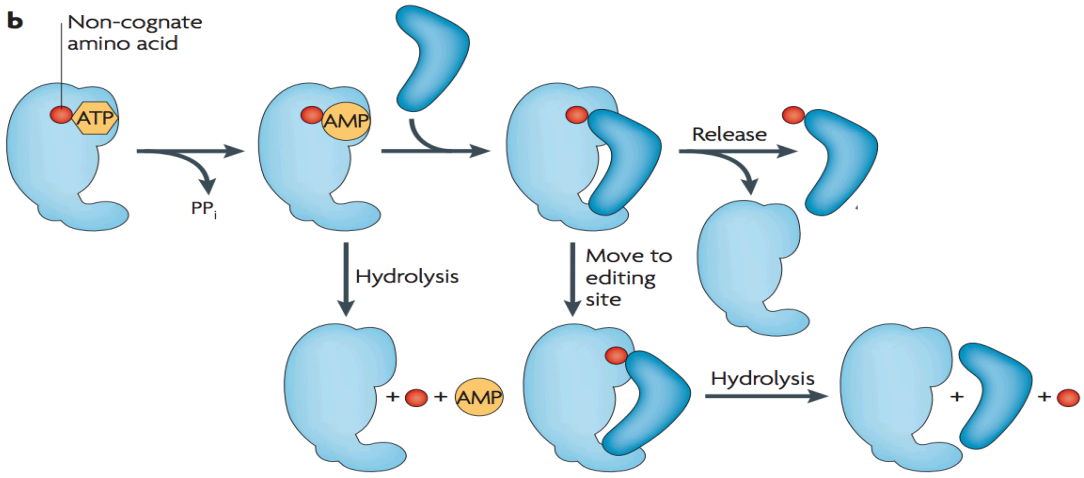

Figure 2: AaRSs are critical in maintaining fidelity in translation because some aaRSs have editing mechanisms to prevent the misincorporation of non-cognate amino acids into proteins. Pre-transfer editing results in the removal of a non-cognate amino acid from the active site of the aaRS; post-transfer editing leads to separation of a non-cognate amino acid from a tRNA (modified from 1).

AaRSs have an important role in preventing errors in translation, and a lack of aaRS quality control has been documented to be detrimental to the cell in both bacteria and eukaryotes. For instance, there is evidence that phenylalanyl-tRNA synthetase (PheRS) quality control is important for the growth of *Escherichia coli* during oxidative stress, where there is an increased concentration of *meta*-Tyrosine (*m*-Tyr). A PheRS editing deficient *E. coli* strain, unable to edit *m*-Tyr-tRNA^{Phe}, had decreased growth compared to a wild-type (WT) strain in the presence of hydrogen peroxide, which increased the concentration of *m*-Tyr in the cell (2). In *Saccharomyces cerevisiae*, PheRS quality control was shown to be important for the Target of Rapamycin (TOR) pathway, which is responsible for sensing cellular nitrogen availability and responding appropriately. A PheRS editing deficient *S. cerevisiae* strain had decreased activity of Gln3p, a transcription factor involved in the TOR pathway, compared to a WT strain when grown in ammonium (3). Additionally, neurodegeneration of Purkinje cells was documented in mice with an editing defective alanyl-tRNA synthetase (AlaRS), due increased amount of

misfolded proteins (4). These examples provide evidence that aaRS quality control is important for cellular viability across all domains of life.

While there are many conditions where aaRS quality control is required, there is also evidence that mistranslation can be beneficial to the cell. For example, in *Candida albicans*, a lack of translational quality control, leading to increased leucine misincorporation, benefits the cell because it can result in new proteins. It can also generate a stress response, aiding in survival in the presence of harmful compounds such as hydrogen peroxide (1). Moreover, increased misincorporation of methionine occurs in mammalian cells under high concentrations of reactive oxygen species (ROS), when pathogens are detected. Under these conditions, mistranslation is beneficial because methionine decreases the damage sustained by cells during oxidative stress (1). This evidence suggests that mistranslation is not always harmful to cells.

Despite the fact that aaRSs can help maintain fidelity during translation, aaRSs have also been shown to mischarge tRNA, which can lead to mistranslation. It has been documented that WT *Streptococcus pneumoniae* isoleucyl-tRNA synthetase (IleRS) can form Val-tRNA^{Ile} and Leu-tRNA^{Ile}. However, *E. coli* IleRS was not able to mischarge tRNA^{Ile} with valine and leucine to the same extent as the *S. pneumoniae* enzyme (5). Moreover, *S. pneumoniae* AlaRS has been shown to mischarge tRNA^{Ala} with serine and glycine, which may have additional ramifications in the cell (6). In *S. pneumoniae*, a Gram-positive bacterium, MurM is involved in peptidoglycan crosslinking; this enzyme is partially responsible for branching of alanine-alanine or serine-alanine from the stem peptide (6). Mischarged Ser-tRNA^{Ala} has been shown to be used by MurM with a higher efficiency than tRNA^{Ala} charged with the cognate amino acid, alanine. MurM is also important in *S. pneumoniae* resistance to penicillin (6). The roles of AlaRS mischarging of tRNA^{Ala} and peptidoglycan crosslinking on penicillin resistance merits further study.

To continue to investigate the role of AlaRS quality control in another Gram-positive bacterium and compare this to Gram-negative bacteria (7), an AlaRS editing deficient *B. subtilis*

strain was made. First, *B. subtilis alaS*, which encodes for AlaRS, was cloned into pET28a (8). Since a C666A substitution has been shown to decrease editing activity in *E. coli* AlaRS, *E. coli* and *B. subtilis* AlaRS amino acid sequences were aligned; it was determined that C668 was the homologous residue in *B. subtilis* AlaRS (9). *alaS* was mutagenized to encode C668A AlaRS. *B. subtilis* WT and C668A AlaRS were isolated using His-tag protein purifications. *B. subtilis* C668A AlaRS was compared *in vitro* to WT AlaRS to ensure that this mutation diminished editing activity. It was found that the C668A substitution did not affect aminoacylation of $tRNA^{Ala}$ with alanine, the cognate amino acid. However, the C668A substitution significantly increased mischarging of $tRNA^{Ala}$ with serine, a non-cognate amino acid, compared to WT AlaRS. C668A AlaRS also showed decreased editing activity of Ser-$tRNA^{Ala}$ compared to WT AlaRS. To study the effects of AlaRS quality control *in vivo*, an AlaRS editing deficient strain of *B. subtilis* was made. C668A *alaS* was inserted into the chromosome of *B. subtilis* using pMiniMad2 allelic replacement. When grown in Luria Broth (LB) and M9 Minimal Media, there were no significant differences between the WT and AlaRS editing deficient *B. subtilis* strains. Additionally, the growth of WT and AlaRS editing deficient *B. subtilis* strains in M9 minimal media with addition of 0.5 mM or 5 mM serine were compared.

Results

Determining a mutation to eliminate *B. subtilis* AlaRS editing activity

First, a mutation to decrease the editing activity of *B. subtilis* AlaRS was identified. It has been previously documented that a C666A substitution in *E. coli* AlaRS leads to mischarging of Ser-$tRNA^{Ala}$ and Gly-$tRNA^{Ala}$ and decreases the enzyme's ability to edit mischarged $tRNA^{Ala}$ (9). To determine if there was a homologous residue to C666 in *B. subtilis* AlaRS, *B. subtilis* and *E. coli* AlaRS protein sequences were aligned using Protein BLAST NCBI (Figure 3). While the sequences were only 45% identical, C668 in *B. subtilis* AlaRS aligned with *E. coli* C666 and

surrounding residues. Site-directed mutagenesis was used to induce a missense mutation in *B. subtilis alaS* to encode the substitution C668A.

Score	Expect	Method	Identities	Positives	Gaps
742 bits(1916)	0.0	Compositional matrix adjust.	400/890(45%)	564/890(63%)	34/890(3%)

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Sbjct  5    TSAEVRQMFLDFFKEKGHAVEPSASLVPHEDPSLLWINSGVATLKKYFDGRVVPENPRIV  64

Query  64   TSQRCVRAGGKHNDLENVGYTARHHTFFEMLGNFSFGDYFKHDAIQFAWELLTSEKWFAL  123
             +Q+ +R     ND+ENVG TARHHTFFEMLGNFS GDYFK +AI +AWE LTS+KW
Sbjct  65   NAQKAIRT----NDIENVGKTARHHTFFEMLGNFSIGDYFKEEAITWAWEFLTSDKWIGF  120

Query  124  PKERLWVTVYESDDEAYEIWEKEVGIPRERIIRIGDNKGAPYASDNFWQMGDTGPCGPCT  183
             KE L VTV+  D+EAYE W K++GIP ERIIR+           NFW +G+ GP GP T
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Sbjct  230  GLERMVSVIQNVPTNFDTDLFVPIIKATESISGETYGKDNVKDTAFKVIADHIRTVAFAV  289

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            +DG +PSNE RGYVLRR++RRAVR+   +     F + LV  + ++M     ++K +
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Sbjct  589  SLVTENRLRFDFSHFGQVTKEELEQIERIVNEKIWASIPVSIDLKPIAEAKEMGAMALFG  648

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```

Figure 3: Alignment of *E. coli* (Query) and *B. subtilis* (Subject) AlaRS amino acid sequences. The homologous cysteine residues being compared, C666 in *E. coli* AlaRS and C668 in *B. subtilis* AlaRS, are boxed in blue.

Cloning and Purification of *B. subtilis* WT and C668A AlaRS

The gene which encodes for AlaRS, *alaS*, was PCR amplified from *B. subtilis* genomic DNA, inserted into pET28a and transformed into BL21(DE3) *E. coli* after the insertion was confirmed with Sanger sequencing (8). Then, a missense mutation encoding C668A AlaRS was

introduced into *alaS* using site-directed mutagenesis. pET28a *alaS* was used as the template DNA plasmid. After confirmation of the mutation with Sanger sequencing, pET28a C668A *alaS* was transformed into BL21(DE3) *E. coli*.

His-tag protein purifications were used to isolate *B. subtilis* WT and C668A AlaRS for characterization *in vitro*. *E. coli* containing pET28a *alaS* or C668A *alaS* were grown to an OD_{600} of 0.3-0.6. 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures to induce expression of *alaS* in pET28a. The cells were grown for an additional four hours and then pelleted. After sonication to lyse the cells and centrifugation, Talon metal affinity columns were used to purify AlaRS. SDS-PAGE gels (Figures 4 and 5) were used to assess the presence and purity of AlaRS isolated in the elution fractions. According to UniProt, *B. subtilis* AlaRS is about 97 kDa; therefore, the isolated protein should run at about 100 kDa on an SDS-PAGE gel. In the elution fractions for both WT (Figure 4) and C668A (Figure 5) AlaRS, there was a thick band at approximately 100 kDa. Active site titrations were used to assess activity of WT and C668A AlaRS by monitoring formation of the aminoacyl adenylate. WT AlaRS was found to be approximately 162.6 μM active, while C668A AlaRS was found to be approximately 342.6 μM active.

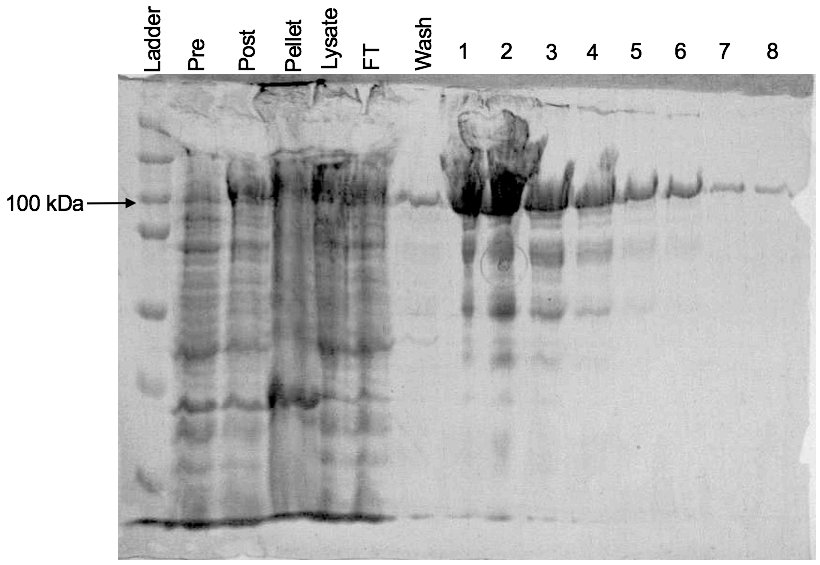

Figure 4: SDS-PAGE gel from the His-tag purification of *B. subtilis* WT AlaRS. From left to right, BioRad Precision Plus Protein Standards Kaleidoscope ladder, pre-induction, post-induction, pellet, lysate, flow through (FT), wash and elution fractions 1-8. *B. subtilis* AlaRS is approximately 97 kDa (UniProt). Elution fractions 1-6 were combined and dialyzed to isolate AlaRS.

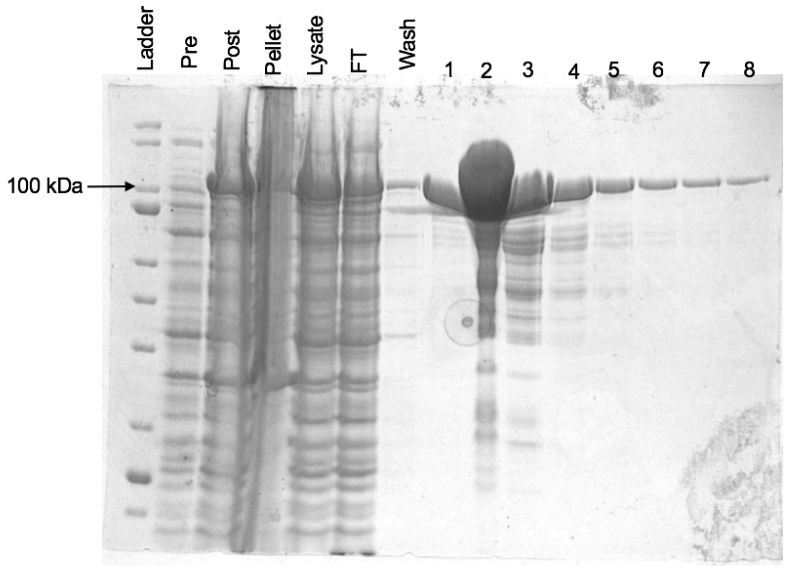

Figure 5: SDS-PAGE gel from the His-tag purification of C668A AlaRS. From left to right, NEB Protein (10-200 kDa) ladder, pre-induction, post-induction, pellet, lysate, FT, wash and elution fractions 1-8. *B. subtilis* AlaRS is approximately 97 kDa (UniProt), and expected to run approximately the same distance as the 100 kDa marker. Elution fractions 1-5 were combined and dialyzed to isolate C668A AlaRS.

B. subtilis WT and C668A AlaRS charge *B. subtilis* $tRNA^{Ala}$ with alanine

Both WT and C668A *B. subtilis* AlaRS were able to charge $tRNA^{Ala}$ with alanine, the cognate amino acid (Figure 6). This suggests that the C668A substitution does not affect the mutant enzyme's active site, since it is still able to aminoacylate $tRNA^{Ala}$. Moreover, this suggests that the C668 residue is not essential for the generation of Ala-$tRNA^{Ala}$. It is important that C668A AlaRS is still able to efficiently charge $tRNA^{Ala}$ with its cognate tRNA to solely determine the effects of AlaRS mischarging of $tRNA^{Ala}$ with non-cognate amino acids in *B. subtilis*. The inability to charge $tRNA^{Ala}$ with alanine may cause different effects in the cell than the mischarging of $tRNA^{Ala}$.

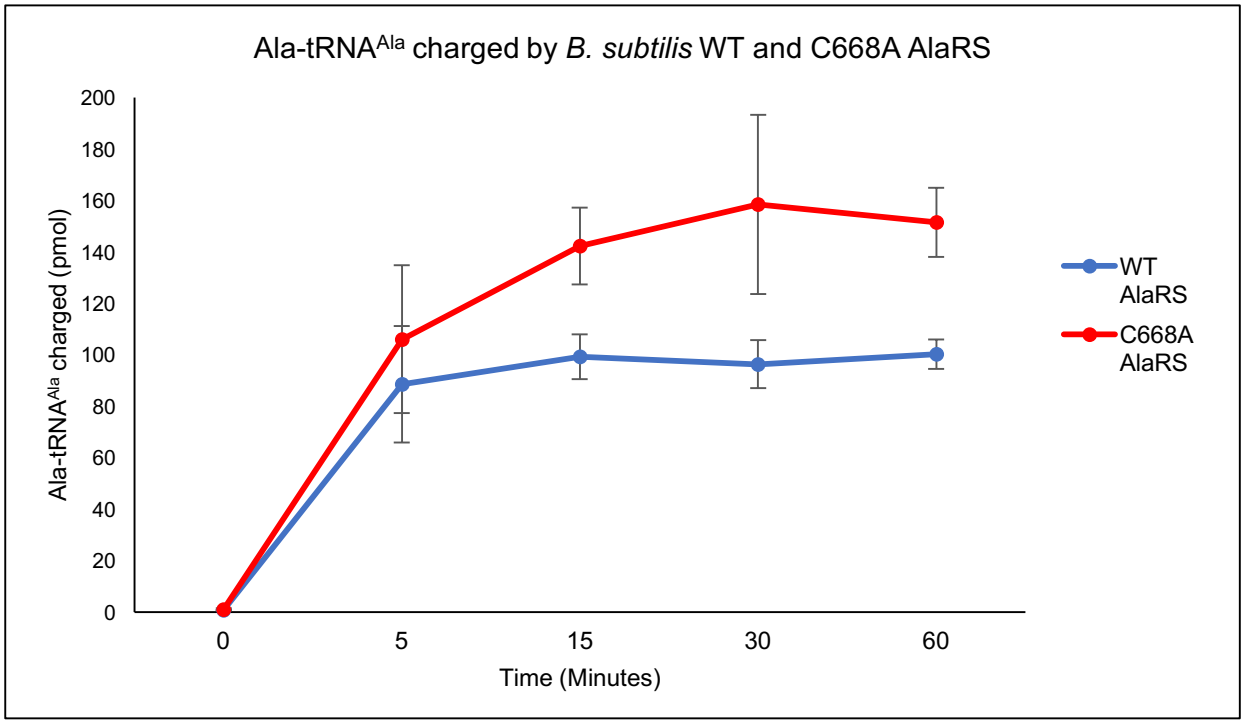

Figure 6: Aminoacylation of *B. subtilis* tRNA^{Ala} with ^{14}C-alanine by *B. subtilis* WT (blue) and C668A (red) AlaRS determined by aminoacylation assays. Time points were taken at 0 (before addition of enzyme), 5, 15, 30 and 60 minutes. Triplicates were performed and averaged for each enzyme. Error bars represent the standard deviation of the triplicates at each time point.

B. subtilis C668A AlaRS mischarges tRNA^{Ala} with serine

B. subtilis C668A AlaRS mischarges tRNA^{Ala} with serine, a non-cognate amino acid (Figure 7). *B. subtilis* WT AlaRS does not mischarge tRNA^{Ala} with serine (Figure 7), indicating its ability to discriminate between the cognate and non-cognate amino acids for tRNA^{Ala} and prevent accumulation of mischarged tRNA. The concentration of Ser-tRNA^{Ala} charged by *B. subtilis* WT AlaRS is similar to the negative control (Figure 7). This indicates that the C668A substitution diminishes the enzyme's ability to properly discriminate between alanine and serine, leading to a high concentration of Ser-tRNA^{Ala} formed. Additionally, this suggests the importance of the C668 residue in AlaRS quality control and prevention of mischarging of tRNA^{Ala} with serine.

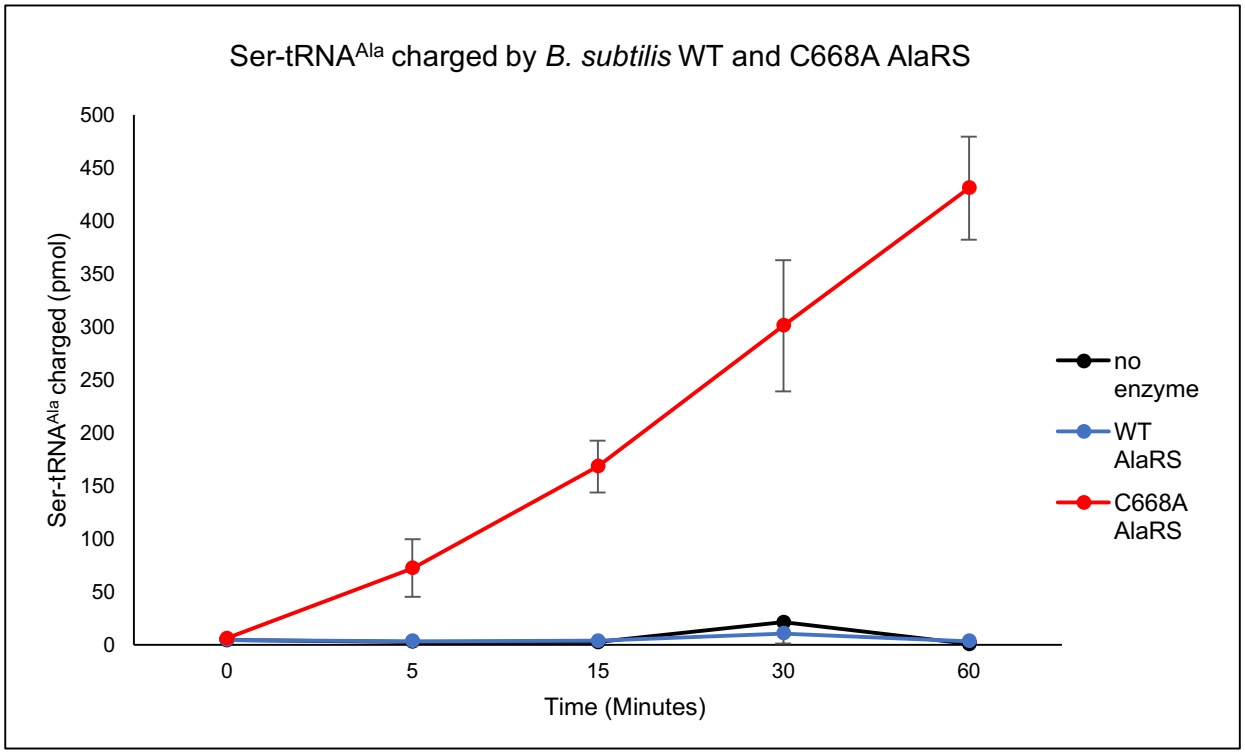

Figure 7: Misacylation of *B. subtilis* tRNA^{Ala} with ^{3}H-serine by *B. subtilis* WT (blue) and C668A (red) AlaRS over time. Time points were taken at 0 (before addition of enzyme), 5, 15, 30 and 60 minutes. Triplicates were performed for each enzyme and averaged. Error bars on the graph represent the standard deviation of the triplicates each time point. Additionally, one experiment was performed without addition of either enzyme as a negative control (black).

B. subtilis C668A AlaRS has diminished ability to edit mischarged Ser-tRNA^{Ala}

B. subtilis C668A AlaRS has a diminished ability to edit tRNA^{Ala} mischarged with serine, and is similar to the no enzyme control (Figure 8). *B. subtilis* WT AlaRS is able to edit Ser-tRNA^{Ala} that has been mischarged, leading to the hydrolysis of the bond between serine and tRNA^{Ala} (Figure 8). When comparing the concentrations of Ser-tRNA^{Ala} remaining at the end of the reactions, it is evident that *B. subtilis* C668A AlaRS is unable to edit and hydrolyze the non-cognate amino acid from tRNA^{Ala} at the same level as WT AlaRS (Figure 8). This suggests the importance of the C668 residue in the ability of AlaRS to edit mischarged tRNA^{Ala}.

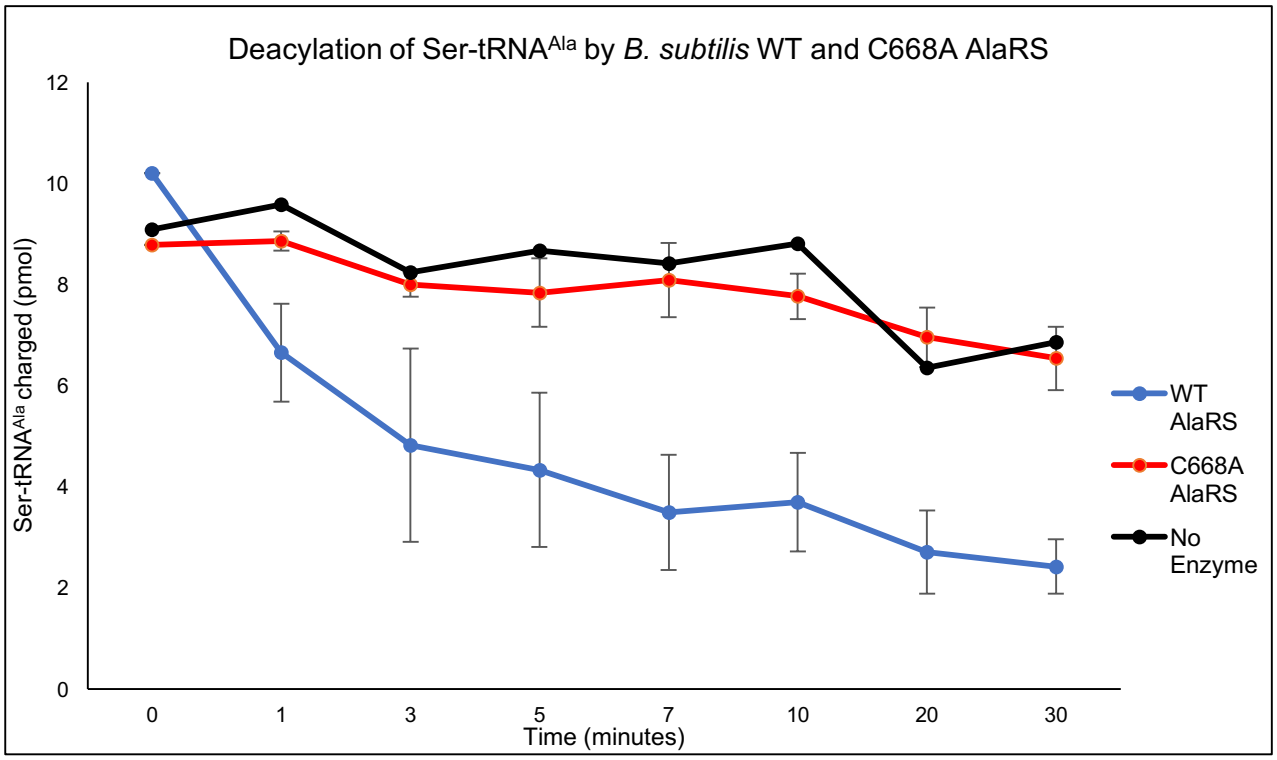

Figure 8: Editing of preformed ^{3}H-Ser-tRNA^{Ala} by *B. subtilis* WT (blue) and C668A (red) AlaRS. Time points were taken at 0 (before enzyme addition), 1, 3, 5, 7, 10, 20 and 30 minutes. Experiments for each enzyme were performed in triplicate. Error bars represent the standard deviation between each replicate at that time point. A negative control without enzyme was also performed (black).

AlaRS quality control is not required for viability of *B. subtilis* in LB or M9 Minimal Media

When grown in LB, there was no significant difference in growth between the WT and AlaRS editing deficient *B. subtilis* strains (Figure 9A). This suggests that AlaRS quality control is not required for growth of *B. subtilis* in LB. Additionally, there was no significant difference between the growth of the WT and AlaRS editing deficient *B. subtilis* strains in M9 minimal media (Figure 9B); this provides evidence that AlaRS quality control is also not required for growth of *B. subtilis* in M9 minimal media.

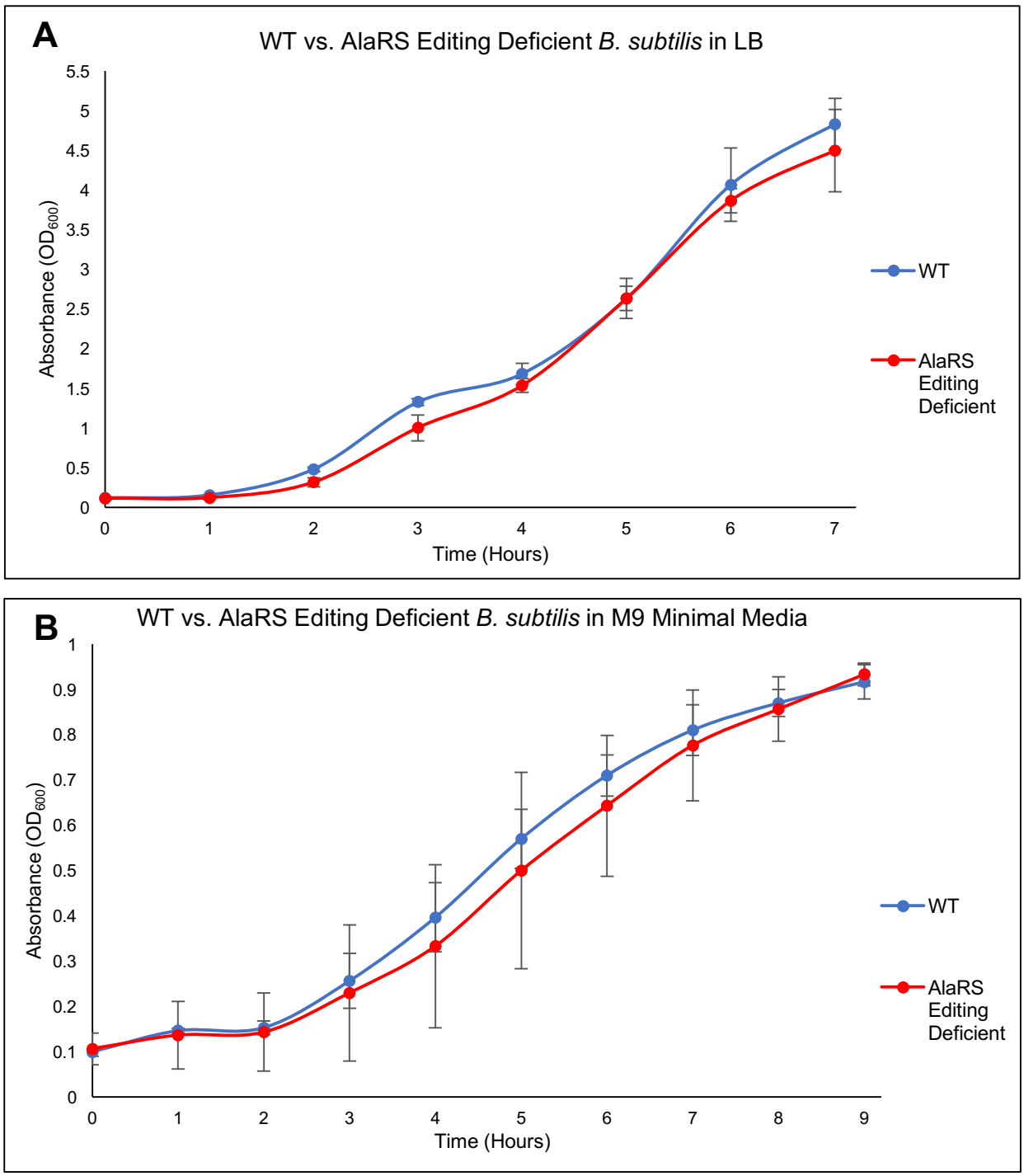

Figure 9: Growth in LB (**A**) and M9 minimal media (**B**) of WT and AlaRS editing deficient strains of *B. subtilis*. Overnight cultures were back diluted to a final OD_{600} of 0.1. Absorbance readings (OD_{600}) of each culture were taken every hour for seven hours (in LB) or nine hours (in M9 minimal media). Biological triplicates were grown for each strain, and the OD_{600} measurements were averaged. Error bars represent the standard deviation of the OD_{600} measurements at each time point.

WT vs. AlaRS Editing Deficient *B. subtilis* in M9 Minimal Media with Excess Serine

WT and AlaRS editing deficient *B. subtilis* strains were grown in M9 minimal media with addition of 0.5 mM or 5 mM serine. After nine hours of growing at 37°C, there was not a significant difference in the average absorbance (OD_{600}) between the two strains in either 0.5 mM or 5 mM serine (Figure 10). However, the difference in growth between the WT and AlaRS editing deficient *B. subtilis* strains in 0.5 mM serine, where the average absorbance (OD_{600}) of the AlaRS editing deficient strain is slightly less than the WT strain, is trending toward serine sensitivity. A concentration of excess serine that inhibits the growth of the AlaRS editing deficient strain more significantly than the WT strain of *B. subtilis* still needs to be determined.

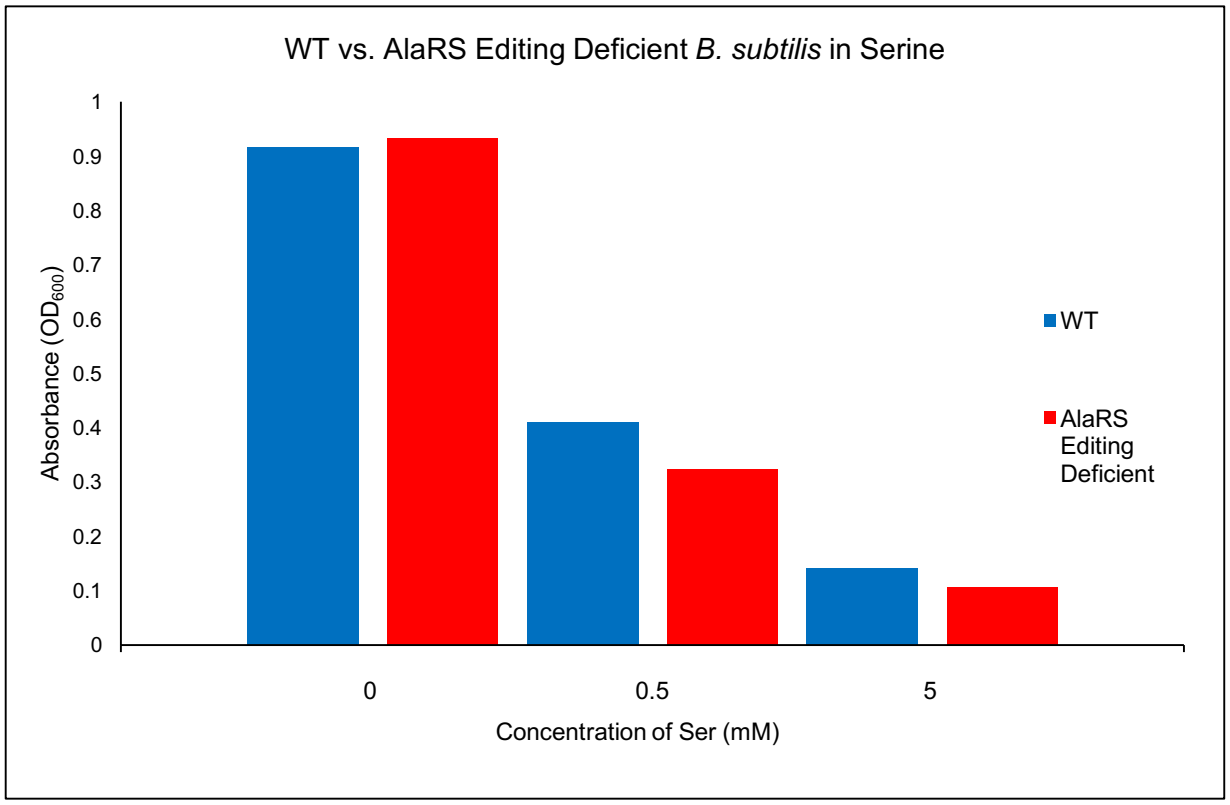

Figure 10: Absorbance (OD_{600}) of WT and AlaRS editing deficient strains of *B. subtilis* after 9 hours of growth at 37°C in M9 minimal media + excess serine (0 mM, 0.5 mM and 5 mM).

Discussion

A key function of some aaRSs, including AlaRS, is their ability to recognize mischarged tRNA and remove the non-cognate amino acid to prevent its misincorporation into proteins (1,10). Since this is a critical role of AlaRS, it is important to understand how lacking AlaRS quality control affects different organisms. To study the role of AlaRS quality control in *B. subtilis*, an AlaRS editing deficient strain was made.

A mutation, C668A, was made in *B. subtilis alaS* to encode for an AlaRS enzyme that had deficient editing activity. This mutation was based on a previously documented C666A AlaRS mutant with decreased editing function in *E. coli* AlaRS (9). *B. subtilis* C668A AlaRS was tested *in vitro* to determine if the mutation decreased editing before making a genomic mutant with the same mutation. It was determined that the C668A substitution did not hinder the ability of AlaRS to charge $tRNA^{Ala}$ with alanine, when compared to WT AlaRS. It was essential that the mutation did not disrupt aminoacylation of the cognate amino acid, to allow the study of the effects of lacking AlaRS editing activity in *B. subtilis*.

C668A AlaRS was able to mischarge $tRNA^{Ala}$ with serine significantly more than WT AlaRS. Additionally, C668A AlaRS had decreased editing of mischarged Ser-$tRNA^{Ala}$ compared to WT AlaRS. This provides evidence that the C668A mutation disrupts the ability of AlaRS to edit non-cognate amino acids from $tRNA^{Ala}$, without hindering its ability to charge Ala-$tRNA^{Ala}$. These results suggest that C668 is a critical residue for AlaRS editing activity in *B. subtilis*. When this cysteine residue is substituted with alanine, substantial Ser-$tRNA^{Ala}$ mischarging occurs and there is diminished editing of mischarged tRNA.

After confirmation that this mutation disrupted AlaRS editing function, C668A *alaS* was inserted into the chromosome of *B. subtilis* using pMiniMad2 allelic replacement to make a AlaRS editing deficient strain. This allowed testing *in vivo* of how lacking AlaRS quality control affects the organism. Previously, a AlaRS editing deficient *E. coli* strain was shown to have substantial growth deficiencies compared to a WT strain in LB and with increasing excess

serine; there was also a slight growth defect observed in the AlaRS editing deficient *E. coli* strain in minimal media (Paul Kelly, unpublished data). However, the AlaRS editing deficient strain of *B. subtilis* did not have growth defects compared to a WT strain in LB or M9 minimal media. This suggests possible differences in the roles of AlaRS quality control in Gram-positive and Gram-negative organisms that should be further explored.

Now that a AlaRS editing deficient *B. subtilis* strain has been made, the effects of lacking AlaRS editing activity can be investigated *in vivo*. In the future, serine sensitivity growth assays can be done to determine which concentration of non-cognate amino acid, if any, inhibits the growth of the editing deficient strain more significantly than the WT strain. Neither a 0.5 mM nor 5 mM serine addition led to significant differences in growth between the two strains. Additionally, the role of AlaRS quality control in sporulation can be studied by comparing sporulation in the WT and AlaRS editing deficient strains; it has been shown that IleRS editing activity in *B. subtilis* is important for sporulation (7). Lastly, since it was previously found that mischarged Ser-tRNA^{Ala} is used more efficiently than tRNA^{Ala} charged with alanine by MurM in *S. pneumoniae*, a Gram-positive organism, the role of AlaRS quality control in peptidoglycan synthesis of other Gram-positive organisms, such as *B. subtilis* (6, 7) should be further explored.

Methods

Alignment of *E. coli* AlaRS and *B. subtilis* AlaRS protein sequences

Amino acid sequences of *E. coli* and *B. subtilis* AlaRS were obtained from UniProt and aligned using Protein BLAST NCBI.

Insertion of *B. subtilis alaS* into pET28a

Using the polymerase chain reaction (PCR), *B. subtilis alaS* was amplified from genomic DNA. The primers (5'-GCCGCGCGGCAGCCATATGAAACACTTAACTTCTGCGGAAGTG-3',

5’-TGGTGGTGGTGGTGCTTATAAAACGGATTTCACCCAATCTTCTACAG-3’) used for PCR generated overhang regions at the ends of *alaS*, to allow subsequent insertion into pET28a. The presence and size of PCR product was confirmed using 0.8% agarose gel electrophoresis. Restriction enzymes *XhoI* and *NdeI* were used to digest pET28a. Success of the restriction digest was confirmed using agarose gel electrophoresis. Gibson assembly was used to insert *alaS* into pET28a. The Gibson assembly reaction was transformed into XLIBlue *E. coli* and the cells were plated onto a LB + 25 µg/mL kanamycin (kan) agar plate. Plasmids were purified from candidates and screened for the *alaS* insertion using a *BlpI* restriction digest. The insertion of *alaS* into pET28a was confirmed with Sanger Sequencing using T7 and T7-Term primers from GENEWIZ. This plasmid was transformed into BL21(DE3) *E. coli* and the cells were plated onto a LB + 25 µg/mL kan agar plate.

Site-directed mutagenesis of *B. subtilis alaS*

Site-directed mutagenesis was used to mutate *alaS*. pET28a *alaS* was purified from BL21(DE3) *E. coli* and used as the template DNA for this reaction. The primers (5’-GATTACAGCTTAGAGCTGGCCGGCGGCTGCCACGTCAG-3’, 5’-CTGACGTGGCAGCCGCCGGCCAGCTCTAAGCTGTAATC-3’) were designed to mutate nucleotides 2002 and 2003 from TG to GC. This mutation results in the amino acid substitution C668A. After QuikChange site-directed mutagenesis, *DpnI* was added to cleave the template plasmid. The product was transformed into XLIBlue *E. coli* and plated onto a LB + 25 µg/mL kan agar plate. The presence of the mutation was confirmed using Sanger sequencing with a primer (5’-CTCATGTCAATCAGGCGGGC-3’). pET28a C668A *alaS* was transformed into BL21(DE3) *E. coli*.

Purification of *B. subtilis* WT and C668A AlaRS

B. subtilis WT and C668A AlaRS were purified using His-tag protein purification. First, BL21(DE3) *E. coli* cells containing either pET28a *alaS* or pET28a C668A *alaS* were grown until an OD_{600} of 0.3-0.6 in LB + 25 µg/mL kan. 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce the expression of pET28a. After four hours, the cells were pelleted and stored at -80^{o}C. The pellet was resuspended in buffer (25 mM Tris, 300 mM NaCl, 10% v/v Glycerol and 5 mM Imidazole). Sonication was used to lyse the cells, using four sets of three 10 second pulses. After centrifugation at 22,000 g for 30 minutes at 4^{o}C twice, the lysate was loaded on a Talon metal affinity resin column. The column was washed with buffer (25 mM Tris, 300 mM NaCl, 10% v/v Glycerol and 5 mM Imidazole) and the protein was eluted with buffer and 250 mM imidazole in 1 mL fractions. Purity of the fractions and presence of target protein were assessed using SDS-PAGE gel electrophoresis. Fractions of protein were combined, concentrated using centrifugation and dialyzed. Activity of *B. subtilis* WT and C668A AlaRS were determined using active site titration assays with ^{14}C-alanine.

Active Site Titration

100 mM Na^{2+} HEPES (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 1 µg pyrophosphatase, 100 µM ^{14}C-Ala and 5 µL AlaRS or storage buffer (negative control) were combined and incubated at 37^{o}C for 10 minutes. Three reactions per enzyme were performed: no enzyme, 1:10 dilution of AlaRS and 1:50 dilution of AlaRS. Protran BA 85 filters were washed with buffer (50 mM Na^{2+} HEPES (pH 7.2), 15 mM KCl, 5 mM $MgCl_2$) over a vacuum manifold. After incubation, the 50 µL reactions were placed on separate filters which were rinsed with buffer three additional times. The filters were dried and placed in a scintillation counter to quantify the concentration of ^{14}C-Ala present in each sample.

Purification of *B. subtilis* tRNA^{Ala}

Two primers were designed using a sequence of *B. subtilis* $tRNA^{Ala}$ and the T7 promoter for T7 RNA polymerase. Equal concentrations of these primers (5'-CGAAATTAATACGACTCACTATAGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCACCA-3', 5'-TGGTGGAGCCTAGCGGGATCGAACCGCTGACCTCCTGCGTGCAAAGCAGGCGCTCTC-3') were combined and were ligated together at 100°C for two minutes. The reaction was slowly cooled to 25°C. This ligation product was amplified using PCR with primers (5'-CGAAATTAATACGAC-3', 5'-TGGTGGAGCCTAGCGGGATCG-3'). The resulting PCR product was purified using NucleoSpin Gel and PCR Clean-up Kit.

This purified PCR product was used as the template DNA for an *in vitro* transcription. 40 mM Tris (pH 8.0), 2 mM spermidine, 22 mM $MgCl_2$, 5 mM DTT, 50 µg/mL Bovine Serum Albumin (BSA), 37 µg/mL T7 RNA polymerase, 20 mM 5' Guanosine monophosphate (GMP), 5 µg pyrophosphatase, 4 mM NTPs, RNase inhibitor and template DNA were combined and incubated at 42°C overnight. To purify the *in vitro* transcription reaction, column purification of tRNA was used. The reaction was loaded onto a DEAE sephacel resin column, which was washed and the product was eluted with 1M NaCl. A nanodrop was used to determine the concentration of RNA in these fractions. Fractions were combined and tRNA was isolated using sodium acetate and ethanol precipitation. The concentration of tRNA was assessed using a nanodrop. An aminoacylation assay using *B. subtilis* WT AlaRS and ^{14}C-alanine was used to determine the active concentration of $tRNA^{Ala}$.

In vitro aminoacylation assays

To monitor [Ala-$tRNA^{Ala}$] and [Ser-$tRNA^{Ala}$] charged over time by WT and C668A AlaRS, *in vitro* aminoacylation assays were performed. 100 mM Na^{2+} HEPES (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 1 µg pyrophosphatase, 1 mM DTT, 15 µM *B. subtilis* $tRNA^{Ala}$, 30 µM ^{14}C-alanine or 300 µM ^{3}H-serine and 0.5 µM *B. subtilis* WT or C668A AlaRS were incubated at

37°C for 60 minutes. During the reaction, 9 µL were spotted onto filters at 0 (before enzyme addition), 5, 15, 30 and 60 minutes. The filters were pre-soaked with 5% Trichloroacetic acid (TCA). After the reaction was complete, the filters were washed three times with 5% TCA and once with 95% ethanol. Lastly, the filters were dried and assessed using a scintillation counter. For the aminoacylation assays with serine, a negative control without enzyme was performed by substituting enzyme with protein storage buffer.

Deacylation assay with Ser-tRNA^{Ala}

First, ^{3}H-Ser-tRNA^{Ala} was formed to use in the deacylation assay. 100 mM Na^{2+} HEPES (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 2 µg pyrophosphatase, 1 mM DTT, 15 µM *B. subtilis* tRNA^{Ala}, 150 µM ^{3}H-serine and 0.5 µM *B. subtilis* C668A AlaRS were incubated at 37°C for 60 minutes. Acid phenol chloroform precipitation, with subsequent sodium acetate and ethanol precipitation and centrifugation was used to isolate ^{3}H-Ser-tRNA^{Ala}. Pelleted ^{3}H-Ser-tRNA^{Ala} was resuspended in 100 mM sodium acetate.

Next, the deacylation assays were performed with ^{3}H-Ser-tRNA^{Ala}. 100 mM Na^{2+} HEPES (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, ^{3}H-Ser-tRNA^{Ala}, and 2.5 nM *B. subtilis* WT or C668A AlaRS were incubated for 30 minutes at 37°C. A no enzyme control was performed once using protein storage buffer in place of enzyme. During the reaction, 9 µL was spotted on 5% TCA pre-soaked filters at 0 (before enzyme addition), 1, 3, 5, 7, 10, 20 and 30 minutes. The filters were subsequently washed with 5% TCA three times and 95% ethanol once. The filters were dried and assessed via a scintillation counter.

Insertion of *B. subtilis* C668A *alaS* into pMiniMad2

pMiniMad2 was digested with *SmaI* and the success of the reaction was confirmed with agarose gel electrophoresis. *B. subtilis* C668A *alaS* was PCR amplified with overhang regions for pMiniMad2 using pET28a C668A *alaS* as the template DNA and primers (5'-

ACTCTAGAGGATCCCCATGAAACACTTAACTTCTGC-3', 5'-TCGAGCTCGGTACCCTTATAAAACGGATTTCACCCA-3'). Gibson assembly was used to ligate C668A *alaS* and pMiniMad2. This reaction was transformed into XLIBlue *E. coli* and plated onto a LB + 100 µg/mL ampicillin (amp) agar plate. The plasmids from the candidates were screened using a restriction digest with *XhoI* and subsequent agarose gel electrophoresis. The insertion of C668A *alaS* was confirmed using Sanger sequencing with primers (5'-GCGGATAACAATTTCACACA-3', 5'-CATCAGGCGCCATTCGC-3'). This plasmid was transformed XLIBlue and TGI *E. coli*.

Transformation of pMiniMad2 + C668A *alaS* into *B. subtilis*

A colony of competent (ComI Q12L) *B. subtilis* was grown at 37°C for 4.5 hours in 1x MC media (100 mM $K_2HPO_4 \bullet 3H_2O$, 100 mM KH_2PO_4, 2% glucose, 3 mM trisodium citrate, 22 mg/L ferric ammonium citrate, 0.1% casein hydrolysate, 0.2% potassium glutamate). pMiniMad2 C668A *alaS*, isolated from TGI *E. coli*, was transformed into *B. subtilis* at a 1:100 ratio plasmid: culture. The culture was grown for 2.0 hours at 37°C and then plated on a MLS (25 µg/mL lincomycin and 1 µg/mL erythromycin) agar plate.

Allelic replacement of WT *alaS* with C668A *alaS* to make *B. subtilis* genomic mutant

pMiniMad2 C668A *alaS* was transformed into *B. subtilis* as described above and plated on a MLS agar plate. Then, the resulting colonies were streaked on MLS agar plates and grown overnight at 37°C. One of the colonies from the MLS plates was used to inoculate 3 mL LB. This culture was grown at 25°C overnight. The next day, this culture was diluted, plated on LB and these plates were incubated at 37°C. 100 of these colonies were patched onto LB and then MLS agar plates, which were grown at 37°C overnight. The plates were screened for colonies which could grow on LB, but could not grow on MLS agar plates. Genomic DNA was purified from MLS sensitive colonies using Wizard Genomic DNA Purification kit. *alaS* was PCR

amplified from the genome with primers (5'-GCCGCGCGGCAGCCATATGAAACACTTAACTTCTGCGGAAGTG-3', 5'-TGGTGGTGGTGGTGCTTATAAAACGGATTTCACCCAATCTTCTACAG-3') using genomic DNA of the candidates. The presence of the mutation was confirmed using Sanger sequencing with a primer (5'-CTCATGTCAATCAGGCGGGC-3'). The *alaS* mutation was subsequently confirmed with a colony PCR to amplify *alaS* using primers (5'-ATGAAACACTTAACTTCTGCGG-3', 5'-TTATAAACGGATTTCACCCAATCTTCTAC-3') These PCR products were sent for Sanger sequencing with a primer (5'-CTCATGTCAATCAGGCGGGC-3').

Growth analysis in LB, M9 minimal media and M9 minimal media with serine

The OD_{600} measurements for overnight cultures of either LB or M9 minimal media (glucose, without thiamine) of WT and AlaRS editing deficient *B. subtilis* strains were taken and the cultures were back diluted to a final OD_{600} of 0.1 in either LB, M9 minimal media or M9 minimal media with addition of serine (0.5 mM or 5 mM). OD_{600} measurements were taken for each culture after inoculation and each hour after for 7-9 hours.

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